Piroplasmid vaccine

The invention relates to a Piroplasmid protein or an immunogenic fragment of said protein, to a nucleic acid encoding said Piroplasmid protein or said immunogenic fragment, to cDNA fragments, recombinant DNA molecules and live recombinant carriers comprising said nucleic acid, to host cells comprising said cDNA fragments, recombinant DNA molecules and live recombinant carriers, to vaccines comprising a Piroplasmid protein or an immunogenic fragment of said protein, to methods for the preparation of such vaccines, to the use of such proteins or fragments, and to diagnostic tests.

Babesiosis is a disease, which has a geographically focal occurrence. The reason for this is that the pathogen is transmitted by ticks that feed on a certain reservoir of parasites present in a vertebrate population. Only where ticks are present, Babesiosis can occur. On balance, particularly in indigenous animals, the parasite coexists with the host without causing significant disease. In many cases Babesiosis becomes a problem because of man's activities through inbreeding of genetic traits and/or transporting animals to unfamiliar environments where Babesiosis is endemic (Callow, L.L. and Dalgliesh, R.J., 1982, in: "Immunology of Parasitic Infections", Cohen, S. and Warren, K.S. eds., p. 475-526, Blackwell Scientific).

Babesiosis also holds a threat as zoonotic agent for humans, not only to immunocompromised humans (Gray *et al.*, 2002, Int. J. Med. Microbiol., vol. 291, p. 108-11).

Signs of disease in naturally acquired Babesiosis usually begin 7-21 days after infection. These symptoms include: fever, anorexia, depression, anaemia, haemoglobinuria and rapidly developing weakness. Increased lacrimation, salivation and muscle tremor commonly occur. Nervous signs may develop in terminal infections, and death may occur when the disease is left untreated. Coagulation disturbances lead to increased erythrocyte-stickiness. As a result the blood passage through the microvasculature is hampered, resulting in congestion of internal organs and decreased packed cell volumes (PCV). Also rupture of infected erythrocytes causes loss of large numbers of erythrocytes. These effects impair the oxygen supply to several tissues and subsequently lead to tissue damage as a result of anoxia.

Species from the Babesiidae have now been detected to infect most mammalian species of veterinary importance (Kuttler, K.L., in M. Ristic ed.: "Babesiosis of domestic

animals and man". CRC Press, Inc., Boca Raton, FL, 1988): Cow (*B. divergens, B. bovis, B. bigemina*), Swine (*B. trautmanni, B. perroncitoi*), Sheep (*B. ovis, B. motasi*), Horse (*B. equi, B. caballi*), Dog (*B. canis, B. rossi, B. vogeli*), and Cat (*B. felis, B. cati*). In all these species death or more or less severe economical losses (reduction in quality or quantity of meat, milk, wool, or offspring), or severe reduction in well-being are caused either as a result of the Babesia infection directly, or through facilitation of secondary infections.

Closely related to Babesia are Theileria parasites. These also belong to the taxonomic group of the Piroplasmida, and show many biological and epidemiological relationships to Babesia. Well known Theileria species of veterinary importance are *T. parva*, *T. annulata*, and *T. sergenti*.

Medications exist to cure an established Babesia or Theileria infection, for instance dogs, horses and cows can be treated with imidocarb dipropionate. However such an injection is painful due to tissue irritation. Further it suffers the drawbacks common to such anti-parasitics: the prevention of a build up of immunological memory, potential toxicity, and possible build up of resistance.

It has been shown that Babesiosis and Theileriosis can be controlled by vaccination with live vaccines (reviewed in: Jenkins, M. 2001, Vet Parasitol., vol. 101, p. 291-310). Such vaccines are produced by harvesting erythrocytes from infected animals. For some but not all Babesia species *in vitro* erythrocyte cultures have been developed, to increase the number of parasites. The infected erythrocytes from the animal or the cultures, also known as "stabilates", are then used to vaccinate animals.

Stabilates for Theileria are produced in a similar fashion. In fact, because the need for an effective vaccine is so high, Theileria stabilates have even been produced from the salivary glands of infected ticks.

General disadvantages of such live parasitic vaccines are that the inoculation material is largely uncontrolled, highly variable in its composition, biologically unsafe, and on the whole the process is unethical through the use of a large number of experimental animals. Additionally, Piroplasmid parasites are very unstable; they must be kept away from free oxygen or will die quickly.

Alternatively, not the parasite-infected erythrocytes themselves are used for vaccination, but the serum from the infected host, or the supernatant of an *in vitro* culture. Such surrounding liquids of infected erythrocytes contain so-called Soluble Parasite Antigens (SPA). Little is known about the composition of these preparations. It has been

suggested that the protective activity is due to the immunising capacity of antigens of the merozoite surface coat in the serum or medium, a structure that is left behind during the process of invasion of the erythrocyte (Ristic, M. and Montenegro-James, S., 1988, in: "Babesiosis of Domestic Animals and Man", Ristic, M. ed., p. 163-190, CRC Press). In addition, during *in vitro* culture a number of parasites die, thereby (internal) parasitic antigens are released into the culture medium.

Such SPA preparations are capable of inducing an immune response that, although not necessarily affecting the parasite, sufficiently reduces the clinical manifestations of infection (Schetters and Montenegro-James, S., 1995, Parasitology Today, vol. 11, p. 456-462). For instance SPA from culture supernatant of an *in vitro* culture of *Babesia canis* parasite infected erythrocytes (Pirodog®) induces immunity against homologous (but not to heterologous) challenge infection.

In general, SPA based vaccines bear the same disadvantages as the live parasitic vaccines do, in that they are largely uncharacterised, highly variable and require many precautions to be biologically safe. Additionally the production of such vaccines is very difficult to scale up, as that requires the infection, housing and harvesting from samples of experimental animals to provide parasites, erythrocytes, and/or serum.

It is an object of the invention to provide proteins and fragments thereof that can serve in effective vaccines for prevention or amelioration of infection with a Piroplasmid organism, that are well defined, safe, stable, and with a production that is easy to scale up.

It was surprisingly found now that a vaccine comprising one or more of five novel Piroplasmid proteins, or an immunogenic fragment of one or more of said proteins incorporates all these advantageous characteristics.

Many disadvantages of live parasite- and SPA vaccines can now be overcome by the use of such a Piroplasmid protein or of an immunogenic fragment of said protein in vaccines. Such a protein is highly defined, biologically safe, the product can be stabilized much better than whole live parasites, and its production can be easily scaled up

It was surprisingly found that antibodies raised against Piroplasmid proteins or immunogenic fragments of said proteins, effectively inhibited the invasion of parasites into host cells, and thereby interfered with the parasites' infection cycle. The proteins are therefore called: invasion inhibiting antigen (IIA).

The process of the invasion by a Piroplasmid parasite of its host cell is one of the critical steps in the establishment of parasitic infection. By interfering at this level through induction of antibodies that interfere with this step, the initial entry of parasites into the cells of the host is inhibited. This prevents, or at least diminishes, the level of infection or the clinical signs of disease in a host, and consequently the severity of disease. Also the further spread of the disease in the environment is halted or diminished because less ticks will become carriers when feeding on vaccinated hosts, *ergo* the infection pressure in the environment is decreased.

Piroplasmid IIA's, which can induce protective immune responses that lead to antibodies that inhibit Piroplasmid parasite invasion, can be detected in Piroplasmid parasites, in cultures of proliferating parasites, and in infected cells by specific antisera. These specific antisera recognize these IIA also in 1-D and 2-D (2 dimensional) Western blots of lysates of infected cells, of parasites or their cultures.

The Piroplasmid IIA's can be expressed in an expression system. Proteins, or their fragments, expressed in this way can be used to formulate a vaccine which protects mammalians from disease or its clinical signs upon infection by a Piroplasmid organism, through the induction of specific antibodies or antigen-specific lymphocytes.

Therefore the invention provides a Piroplasmid protein characterised in that said protein comprises an amino acid sequence having a similarity of at least 70%, preferably 75 %, more preferably 80, 85, 90, 92, 94, 95, 96, 97, 98, 99, or 100 % similarity in that order of preference, with the amino acid sequence depicted in SEQ ID NO: 2 or 4, or an immunogenic fragment of said protein.

The invention also provides a Piroplasmid protein characterised in that said protein comprises an amino acid sequence having a similarity of at least 70%, preferably 75 %, more preferably 80, 85, 90, 92, 94, 95, 96, 97, 98, 99, or 100 % similarity in that order of preference, with the amino acid sequence depicted in SEQ ID NO: 6 or 8, or an immunogenic fragment of said protein.

The invention additionally provides a Piroplasmid protein characterised in that said protein comprises an amino acid sequence having a similarity of at least 70%, preferably 75 %, more preferably 80, 85, 90, 92, 94, 95, 96, 97, 98, 99, or 100 % similarity in that order of preference, with the amino acid sequence depicted in SEQ ID NO: 10, or an immunogenic fragment of said protein.

Typical examples of the Piroplasmid proteins of the invention are:

- Piroplasmid IIA number 1 from *Babesia bovis* (BIIA1) the amino acid sequence of which is presented in SEQ ID NO: 2;
- Piroplasmid IIA number 1 from *Theileria annulata* (TIIA1) the amino acid sequence of which is presented in SEQ ID NO: 4;
- Piroplasmid IIA number 2 from *B. bovis* (BIIA2) the amino acid sequence of which is presented in SEQ ID NO: 6;
- Piroplasmid IIA number 2 from *T. annulata* (TIIA2) the amino acid sequence of which is presented in SEQ ID NO: 8;
- Piroplasmid IIA number 3 from *B. bovis* (BIIA3) the amino acid sequence of which is presented in SEQ ID NO: 10.

The term "protein" is meant to incorporate a molecular chain of amino acids. A protein is not of a specific length, structure or shape and can, if required, be modified *in vivo* or *in vitro*, by, e.g. glycosylation, amidation, carboxylation, phosphorylation, or changes in spatial folding. *Inter alia*, peptides, oligopeptides and polypeptides are included within the definition of protein. A protein can be of biologic and/or of synthetic origin.

A "Piroplasmid protein" according to the invention is a protein, which is obtainable from an organism of the Piroplasmids.

Preferably the Piroplasmid protein is obtainable from an organism selected from the group consisting of the species *Babesia divergens, B. bovis, B. motasi, B. caballi, B. equi, B. canis, B. rossi, B. vogeli, B. felis, B. cati, B. ovis, B. trautmanni, B. bigemina, B. microti, B. gibsoni, Theileria annulata, T. parva, T. equi, T. felis, T. canis* and *T. sergenti.*

More preferably the Piroplasmid protein is obtainable from an organism selected from the group consisting of the species *Babesia bovis, , B. caballi, B. equi, B. canis, B. rossi, B. bigemina, Theileria annulata, T. parva* and *T. equi.*

Even more preferably, the Piroplasmid protein is obtainable from an organism selected from the group consisting of the species *Babesia bovis* and *Theileria annulata.*

Most preferably the Piroplasmid protein is obtainable from *Babesia bovis.*

With respect to the current taxonomic classification, the skilled person will realise this may change over time as new insights lead to reclassification into new or other taxonomic groups. However, as this does not change the protein repertoire of the organism involved, only its classification, such re-classified organisms are considered to

be within the scope of the invention. This is especially relevant for such closely related families as Babesiidae and Theileriidae. For example: *Babesia equi* was recently re-classified as *Theileria equi*.

In order to be antigenic, a fragment of a protein needs to be of a certain length; too small fragments will not be processed by antigen presenting cells to fragments that are able as such to associate with MHC molecules, which association is required for proper antigen presentation to lymphocytes. For MHC I receptor binding an antigen fragment that encompasses the epitope consists of at least 8 – 11 amino acids, and for MHC II receptor binding at least 11 – 15 amino acids (reviewed e.g. by R.N. Germain & D.H. Margulies, 1993, Annu. Rev. Immunol., vol. 11, p. 403-450, in: "The biochemistry and cell biology of antigen processing and presentation"). Protein fragments shorter than this may not be antigenic as such: they need to be coupled to a carrier, such as KLH, BSA or the like, using techniques known in the art. When coupled such short fragments may well be able to induce an immune response that is within the scope of the invention.

For the invention, an "epitope" is that part of an antigenic molecule that reacts with the antigen receptor of a T- and/or B-lymphocyte. An epitope according to the invention will therefore induce and/or activate specific T- and/or B-cells such that these cells give rise to an immune reaction that interferes with the course of an infection or disease. Thus, through such epitopes, a protein can induce antibodies and/or generate an immune response.

An "immunogenic fragment" is understood to be an epitope-containing antigenic fragment of a Piroplasmid protein that has the capability to induce immune responses directed against such Piroplasmid proteins, with the provision that such antibodies are capable of interfering with the process of invasion. It will be explained below how such immunogenic fragments can be found.

An immunogenic fragment of a Piroplasmid protein according to the invention comprises at least 10 amino acids taken from the amino acid sequence of a Piroplasmid protein according to the invention.

Preferably such a fragment comprises 12, 15, 20, 30, 40, 50, 75, 100, 150, 200, or 300 amino acids, in that order of preference, taken from the amino acid sequence of a Piroplasmid protein according to the invention.

For instance an immunogenic fragment of a Piroplasmid protein according to the invention is formed by a part of the protein that lacks the N-terminal signal sequence and/or the C-terminal sequence. Other fragments are for instance those comprising a specific epitope from a Piroplasmid IIA protein. Such epitopes may be determined by the methods outlined below. All such immunogenic fragments are within the scope of the invention.

Identification of immunogenic fragments and/or epitopes of a Piroplasmid protein according to the invention, can be easily performed by a variety of straightforward techniques, for instance by the so-called PEPSCAN method, or via computer algorithms that make comparisons to known fragments and/or epitopes.

The PEPSCAN method (WO 84/03564, and WO 86/06487, and H. Geysen *et al.*, Proc. Natl. Acad. Sci. USA 1984, vol. 81, p. 3998-4002, and J. of Immunol. meth. 1987, vol. 102, p. 259-274), is an easy to perform, quick and well-established method for the detection of immunologic determinants of a protein. It comprises the synthesis of a series of peptide fragments progressively overlapping the protein under study, and subsequent testing of these polypeptides with specific antibodies to the protein to identify which of these are able to bind to the antigen receptor of T- and/or B-lymphocytes. Such antibodies to the proteins according to the invention can be obtained by making polyclonal or monoclonal antibodies, by using techniques well known in the art.

The use of computer algorithms in the designation of specific protein fragments as the immunologically important epitopes on the basis of their sequential and/or structural agreement with epitopes that are known, is also a well-known technique. The determination of these regions can be based on a combination of the hydrophilicity criteria according to Hopp and Woods (Proc. Natl. Acad. Sci. USA 1981, vol. 78, p. 3824-3828), and the secondary structure aspects according to Chou and Fasman (Advances in Enzymology 1987, vol. 47, p. 45-148, and US patent 4,554,101). Immunogenic epitopes can likewise be predicted from the protein's amino acid sequence by computer with the aid of Berzofsky's amphiphilicity criterion (, Science 1987, vol. 235, p. 1059-1062 and US patent application NTIS US 07/005,885). A condensed overview of the use of these methods is found in Shan Lu (common principles: Tibtech 1991, vol. 9, p. 238-242), Lu (review: Vaccine 1992, vol. 10, p. 3-7), and Berzofsky (HIV-epitopes; 1991, The FASEB Journal, vol. 5, p. 2412-2418).

An illustration of the effectiveness of using these methods was published by H. Margalit *et al.* (J. of Immunol. 1987, vol. 138, p. 2213-2229) who describe success rates of 75 % in the prediction of T-cell epitopes using such methods. Still further proof is the

successful prediction of the 6 antigenic peptides from BIIA1 and BIIA2, as outlined in Example 1, section 1.1.5.

Subsequently, it has to be determined if an epitope found using the methods described above is indeed capable of interfering with the process of invasion. This can however be done very quickly and easily in a simple *in vitro* invasion inhibition experiment. Such an experiment is described in Example 1.1.11.

The percentage of similarity of an amino acid sequence with a protein according to the invention must be determined by amino acid alignment to the full-length amino acid sequence of SEQ ID NO: 2, 4, 6, 8, or 10.

The percentage of similarity with a protein according to the invention must be determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BlastP" (T. Tatusova & T. Madden, 1999, FEMS Microbiol. Letters, vol. 174, p. 247-250), that can be found.at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. The comparison-matrix that is used is: "Blosum62", with the default parameters: open gap penalty: 11; extension gap penalty: 1; and gap x_dropoff: 50.

This program lists the percentage of amino acids that are identical as "Identities", and the percentage of amino acids that are similar as "Positives". "Similar" amino acids are those amino acids that are identical plus those that are equivalent; "equivalent" amino acids are described below.

It will be understood that, for a particular Piroplasmid protein, natural variations exist between the proteins associated with individual strains or species of Piroplasmids. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. Amino acid substitutions, which do not essentially alter biological and immunological activities, have been described, e.g. by Neurath *et al.* (1979, in: "The Proteins", Academic Press New York). Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, i.a. Ser/Ala, Ser/Gly, Asp/Gly, Asp/Asn, Ile/Val (see Dayhof, M.D., 1978, "Atlas of protein sequence and structure", Nat. Biomed. Res. Found., Washington D.C. vol. 5, suppl. 3). Other common amino acid substitutions include Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Thr/Phe, Ala/Pro, Lys/Arg, Leu/Ile, Leu/Val and Ala/Glu. Such related and commonly substituted amino acids are termed "equivalent". Based on this information, Lipman and Pearson developed a method for rapid and sensitive protein comparison

(Science 1985, vol. 227, p. 1435-1441) and determining the functional similarity between proteins. Such amino acid substitutions of the exemplary embodiments of this invention, as well as variations having deletions and/or insertions are within the scope of the invention as long as the resulting proteins retain the capability of inducing immune responses that inhibit Piroplasmid parasite proliferation, for instance antibodies that inhibit Piroplasmid parasite invasion. Such variations in the amino acid sequence of a certain Piroplasmid protein according to the invention are considered as "biological- or functional homologs", and are all within the scope of the invention.

This explains why a Piroplasmid protein according to the invention, when isolated from different Piroplasmid species, may have a similarity down to 70 % with for example the amino acid sequences depicted in SEQ ID NO: 2, 4, 6, 8, or 10 while still representing the same protein with the same characteristics, in the example presented: to be able to induce antibodies that inhibit Piroplasmid parasite invasion.

When comparing Piroplasmid proteins according to the invention amongst themselves, Piroplasmid proteins according to the invention obtained from different Piroplasmid organisms typically have over 50 % amino acid similarity; when obtained from different Babesia species, such proteins typically have over 85 % amino acid similarity, and when obtained from different isolates from *B. bovis*, such proteins typically have over 95 % amino acid similarity.

The preferred way to produce the Piroplasmid proteins according to the invention is by using genetic engineering techniques and recombinant expression systems. These may comprise using nucleic acids, cDNA fragments, recombinant DNA molecules, live recombinant carriers, and/or host cells.

Therefore, another aspect of the invention relates to a nucleic acid, characterised in that said nucleic acid encodes a Piroplasmid protein according to the invention, or an immunogenic fragment of said protein.

In an embodiment the nucleic acid according to the invention comprises the nucleic acid sequence depicted in SEQ ID NO: 1, 3, 5, 7, or 9.

The term "nucleic acid" is meant to incorporate a molecular chain of desoxy- or ribonucleic acids. A nucleic acid is not of a specific length, therefore polynucleotides, genes, open reading frames (ORF's), probes, primers, linkers, spacers and adaptors,

consisting of DNA and/or RNA, are included within the definition or nucleic acid. A nucleic acid can be of biologic and/or synthetic origin. The nucleic acid may be in single stranded or double stranded form. The single strand may be in sense or anti-sense orientation. Also included within the definition are modified RNAs or DNAs. Modifications in the bases of the nucleic acid may be made, and bases such as Inosine may be incorporated. Other modifications may involve, for example, modifications of the backbone.

The term "encodes" is meant to incorporate: providing the possibility of protein expression, i.a. through transcription and/or translation when brought into the right context.

A nucleic acid according to the invention encodes a Piroplasmid protein according to the invention, or encodes an immunogenic fragment of said protein.

A nucleic acid according to the invention has a minimal length of 30 nucleotides. Preferably a nucleic acid according to the invention comprises 40, 50, 100, 250, 500, 1000, or 1500 nucleotides in that order of preference.

A nucleic acid according to the invention for instance is a nucleic acid encoding a Piroplasmid protein according to the invention that lacks the N-terminal signal sequence and/or the C-terminal sequence. Other nucleic acids may comprise a sequence encoding a specific epitope of a Piroplasmid protein. Such nucleic acids are all within the scope of the invention.

Excluded from the nucleic acids according to the invention are the following sequences:

- with regard to BIIA1 (SEQ ID NO: 1), the EST sequences:
    - B_bovis-11e05.plc
    - B_bovis-344e09.qlc
    - B_bovis-384f06.qlc
    - B_bovis-261d05.qlc
    - B_bovis-5e5.plc
    - B_bovis-373g01.qlc
    - B_bovis-418b06.qlc
    - B_bovis-375d02.qlc
    - B_bovis-407d03.qlc
    - B_bovis-284-f07.qlc

- with regard to BIIA1 (SEQ ID NO: 1), the assembled contigs:
  - Bbovis.CONTIG.1029
  - Bbovis.CONTIG.227

- With regard to BIIA2 (SEQ ID NO: 5) the EST sequences:
  - B_bovis-417g12.qlc
  - B_bovis-376a10.qlc

- with regard to TIIA2 (SEQ ID NO: 7), the assembled contig:
  - gnl|Sanger_5874|Contig1548

- with regard to TIIA1 (SEQ ID NO: 3), the assembled contig:
  - gnl|Sanger_5874|Contig1

The EST and contig sequences regarding BIIA1 and BIIA2 are available through the Internet web page: www.sanger.ac.uk/projects/b_bovis/ .

The contig sequences regarding TIIA1 and TIIA2 are available through the NCBI BLAST server by selecting Apicomplexa from the Internet page: http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi?organism=euk

The percentage of identity between nucleic acids according to the invention is determined with the computer program "BLAST 2 SEQUENCES" by selecting sub-program: "BlastN" (T. Tatusova & T. Madden, 1999, FEMS Microbiol. Letters, vol. 174, p. 247-250), that can be found at www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html. Parameters that are used are the default parameters: reward for a match: +1; penalty for a mismatch: -2; open gap penalty: 5; extension gap penalty: 2; and gap x_dropoff: 50. Unlike the output of the BlastP program described above, the BlastN program does not list similarities, only identities: the percentage of nucleotides that are identical are indicated as "Identities".

It is well known in the art, that many different nucleic acids can encode one and the same protein. This is a result of what is known in molecular biology as "wobble", or the "degeneracy of the genetic code", wherein several codons or triplets of mRNA will cause the same amino acid to be attached to the chain of amino acids growing in the ribosome during translation. It is most prevalent in the second and especially the third base of each

triplet encoding an amino acid. This phenomenon can result in a heterology of about 30% for two different nucleic acids that still encode the same protein. Therefore, two nucleic acids having a nucleotide sequence identity of about 70 % can still encode one and the same protein.

Another approach for deciding if a certain nucleic acid sequence is or is not a nucleic acid sequence according to the invention, relates to the question if that certain nucleic acid sequence does hybridise under stringent conditions to any of the nucleotide sequences depicted in SEQ ID NO: 1, 3, 5, 7, and 9.

If a nucleic acid sequence hybridises under stringent conditions to the nucleotide sequence as depicted in SEQ ID NO: 1, 3, 5, 7, and 9, it is considered to be a nucleic acid sequence according to the invention.

The definition of stringent conditions follows from the formula for the melting temperature Tm of Meinkoth and Wahl (1984, Anal. Biochem., vol. 138, p. 267-284):

$$Tm = [81.5°C + 16.6(\log M) + 0.41(\%GC) - 0.61(\%formamide) - 500/L] - 1°C/1\%mismatch$$

In this formula, M is molarity of monovalent cations; %GC is the percentage of guanosine and cytosine nucleotides in the DNA; L is the length of the hybrid in base pairs; and mismatch is the lack of an identical match.

Stringent conditions are those conditions under which nucleic acid sequences or fragments thereof still hybridise, if they have a mismatch of 30 % (i.e. if they are only 70 % identical) to the nucleic acid sequence as depicted in any of the SEQ ID NO's: 1, 3, 5, 7, and 9.

Nucleic acids encoding the Piroplasmid proteins according to the invention can be obtained from member species of the Piroplasmida.

However in a more preferred embodiment, the nucleic acids encoding a Piroplasmid protein or immunogenic fragments of said protein according to the invention are characterised in that they are obtainable from an organism selected from the group consisting of the species *Babesia divergens, B. bovis, B. motasi, B. caballi, B. equi, B. canis, B. rossi, B. vogeli, B. felis, B. cati, B. ovis, B. trautmanni, B. bigemina, B. microti, B. gibsoni, Theileria annulata, T. parva, T. equi, T. felis, T. canis* and *T. sergenti.*

More preferably the nucleic acids are obtainable from an organism selected from the group consisting of the species *Babesia bovis, , B. caballi, B. equi, B. canis, B. rossi, B. bigemina, Theileria annulata, T. parva* and *T. equi.*

The possibility of species being taxonomically re-classified or described as new species has been discussed above. As this does not change the organism's genome, such reclassified organisms are also within the scope of the invention.

Also within the scope of the invention are Piroplasmid proteins, immunogenic fragments of said proteins and nucleic acids encoding such Piroplasmid proteins or fragments thereof from non-mammalian Piroplasmids, due to the high conservation of the genes and proteins of the Piroplasmid proteins according to the invention. Such related proteins, or their genes may be called paralogs or orthologs.

Nucleic acids encoding a Piroplasmid protein according to the invention can be obtained, manipulated and expressed by standard molecular biology techniques that are well-known to the skilled artisan, and are explained in great detail in standard text-books like Sambrook & Russell: "Molecular cloning: a laboratory manual" (2001, Cold Spring Harbour Laboratory Press; ISBN: 0879695773). One such type of manipulations is the synthesis of a cDNA fragment from RNA, preferably from mRNA that can be isolated from parasites, or parasite- infected cells or -organisms by techniques known in the art.

Therefore, in another aspect, the invention relates to a cDNA fragment according to the invention.

The preferred method of obtaining a cDNA fragment by reverse transcription is through a polymerase chain reaction (PCR) technique. Standard techniques and protocols for performing PCR are for instance extensively described in C. Dieffenbach & G. Dveksler: "PCR primers: a laboratory manual" (1995, CSHL Press, ISBN 879694473).

In a preferred embodiment, the invention relates to a recombinant DNA molecule comprising a nucleic acid according to the invention, or a cDNA fragment according to the invention, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter.

To construct a recombinant DNA molecule according to the invention, preferably DNA plasmids are employed. Such plasmids are useful e.g. for enhancing the amount of DNA-insert, as a probe, and as tool for further manipulations. Examples of such plasmids for cloning are plasmids of the pBR, pUC, and pGEM series; all these are available from several commercial suppliers.

The nucleic acid encoding a Piroplasmid protein according to the invention or an immunogenic fragment of said protein, can be cloned into separate plasmids and be modified to obtain the desired conformation using techniques well known in the art. However they may also be combined into one construct for improved cloning or expression purposes.

Modifications to the coding sequences encoding a Piroplasmid protein according to the invention or an immunogenic fragment thereof may be performed e.g. by using restriction enzyme digestion, by site directed mutations, or by polymerase chain reaction (PCR) techniques.

For the purpose of protein purification or -detection, or improvement of expression level, additional nucleic acids may be added. This may result in the final nucleic acid comprised in the cDNA fragment, or in the recombinant DNA molecule being larger than the sequences required for encoding a Piroplasmid protein. When such additional elements are inserted in frame, these become an integral part of the Piroplasmid protein that is expressed. Such fused proteins are also within the scope of the invention

An essential requirement for the expression of a nucleic acid, cDNA fragment, or recombinant DNA molecule is that these are operably linked to a transcriptional regulatory sequence such that this is capable of controlling the transcription of the nucleic acid, cDNA, or recombinant DNA. Transcriptional regulatory sequences are well known in the art and comprise i.a. promoters and enhancers. It is obvious to those skilled in the art that the choice of a promoter extends to any eukaryotic, prokaryotic or viral promoter capable of directing gene transcription, provided that the promoter is functional in the expression system used.

In a more preferred embodiment, the invention relates to a live recombinant carrier comprising a nucleic acid according to the invention or a cDNA fragment according to the invention, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter, or a recombinant DNA molecule according to the invention.

Such live recombinant carriers (LRC's) are e.g. micro-organisms such as bacteria, parasites and viruses in which additional genetic information has been cloned, in this case a nucleic acid, a cDNA, or a recombinant DNA molecule, encoding a Piroplasmid protein according to the invention or an immunogenic fragment thereof. Target mammalians inoculated with such LRC's will produce an immunogenic response not only against the immunogens of the carrier, but also against the heterologous protein(s) or immunogenic fragment(s) for which the genetic code is additionally cloned into the LRC, e.g. a

sequence encoding a Piroplasmid protein according to the invention, or an immunogenic fragment thereof.

As an example of bacterial LRC's, attenuated *Salmonella* strains known in the art can attractively be used.

Alternatively, live recombinant carrier parasites have i.a. been described by Vermeulen, A. N. (Int. Journ. Parasitol. 1998, vol. 28, p. 1121-1130).

LRC viruses may be used as a way of transporting a nucleic acid into a target cell. Live recombinant carrier viruses are also called vector viruses. Viruses often used as vectors are Vaccinia viruses (Panicali *et al.* 1982, Proc. Natl. Acad. Sci. USA, vol. 79, p. 4927), Herpesviruses (EP 0473210-A2), and Retroviruses (Valerio, D. *et al.* 1989, in: Baum, S.J., Dicke, K.A., Lotzova, E. and Pluznik, D.H. (Eds.), "Experimental Haematology today", Springer Verlag, New York: pp. 92-99).

The technique of *in vivo* homologous recombination, well known in the art, can be used to introduce a recombinant nucleic acid according to the invention into the genome of an LRC bacterium, parasite or virus of choice, capable of inducing expression of the inserted nucleic acid, cDNA or recombinant DNA according to the invention in the host animal.

Bacterial, yeast, fungal, insect, and vertebrate cell expression systems are used as host cells for expression purposes very frequently. Such expression systems are well known in the art and generally available, e.g. commercially through Invitrogen (the Netherlands).

Therefore, in an even more preferred embodiment, the invention relates to a host cell comprising a nucleic acid according to the invention, a cDNA fragment according to the invention, said nucleic acid or said cDNA fragment being under the control of a functionally linked promoter, a recombinant DNA molecule according to the invention, or a live recombinant carrier according to the invention.

A host cell to be used for expression of a Piroplasmid protein according to the invention may be a cell of bacterial origin, e.g. from *Escherichia coli, Bacillus subtilis, Lactobacillus sp.* or *Caulobacter crescentus*, in combination with the use of bacteria-derived plasmids or bacteriophages for expressing the sequence encoding a Piroplasmid protein. The host cell may also be of eukaryotic origin, e.g. yeast-cells in combination with yeast-specific vector molecules, or higher eukaryotic cells, like insect cells (Luckow *et al.*,1988, Bio-technology, vol. 6, p. 47-55) in combination with vectors or recombinant baculoviruses; plant cells in combination with e.g. Ti-plasmid based vectors or plant viral

vectors (Barton, K.A. *et al.*, 1983, Cell, vol. 32, p. 1033); or mammalian cells like Hela cells, Chinese Hamster Ovary cells or Crandell-Rees feline kidney-cells, also with appropriate vectors or recombinant viruses.

Next to these expression systems, plant cell, or parasite-based expression systems are attractive expression systems. Parasite expression systems are e.g. described in the French Patent Application, publication number 2 714 074, and in US NTIS publication no. US 08/043109 (Hoffman, S. & Rogers, W., 1993). Plant cell expression systems for polypeptides for biological application are e.g. discussed in R. Fischer *et al.* (Eur. J. of Biochem. 1999, vol. 262, p. 810-816), and J. Larrick *et al.* (Biomol. Engin. 2001, vol. 18, p. 87-94).

Expression may also be performed in so-called cell-free expression systems. Such systems comprise all essential factors for expression of an appropriate recombinant nucleic acid, operably linked to a promoter that will function in that particular system. Examples are the *E. coli* lysate system (Roche, Basel, Switzerland), or the rabbit reticulocyte lysate system (Promega corp., Madison, USA).

The Piroplasmid protein according to the invention or immunogenic fragments of said protein are very well suited for the production of a vaccine. Such proteins or fragments can be obtained from parasites, or from animals or cells infected with Piroplasmid parasites. However, much more convenient is the use of the nucleic acids encoding the Piroplasmid protein according to the invention or an immunogenic fragment of said protein, in an expression system. This is followed by harvesting the proteins or fragments produced and formulating these into a protein subunit vaccine, e.g. by admixing a Piroplasmid protein according to the invention or an immunogenic fragment of said protein, and a pharmaceutically acceptable carrier.

Therefore, yet another aspect of the invention relates to a vaccine comprising a protein according to the invention or an immunogenic fragment of said protein, a nucleic acid, a cDNA fragment, a recombinant DNA molecule, a live recombinant carrier, or a host cell according to the invention, or a combination thereof, and a pharmaceutically acceptable carrier.

As described above, a Piroplasmid protein or an immunogenic fragment of said protein can advantageously be used for vaccination. It serves either to interfere with Piroplasmid parasite proliferation (e.g. inhibition of host cell invasion), or will induce

protective immune responses (e.g. specific antibodies or activated lymphocytes) that interfere with parasite proliferation, or the clinical signs it produces.

If such proteins or fragments do not produce the desired response on their own, they can be coupled to a carrier such as KLH, BSA or the like, using techniques known in the art.

The coupling of protein or fragments thereof can also be done to enhance or modify the immune response induced. For instance it is common practice to couple protein(-fragment)s to Tetanus toxoid to enhance the response of T-cells. Also specific effector molecules may be added, such as a toxin, to improve the killing of target cells.

Such couplings can be performed

- chemically, by coupling, conjugation or cross-linking, through dehydration, esterification, etc, of the amino acid sequences either directly or through an intermediate structure.
- physically, by coupling through capture in or on a macromolecular structure, or preferably
- by molecular biological fusion, through the combination of recombinant nucleic acid molecules which comprise fragments of nucleic acid capable of encoding each of the two, such that a single continuous expression product is finally produced.

Such molecular engineering techniques are preferred.

An alternative and efficient way of vaccination is by direct vaccination with DNA encoding the relevant antigen or epitope. Direct vaccination with DNA encoding proteins has been successful for many different proteins, as reviewed in e.g. Donnelly *et al.* (The Immunologist 1993, vol. 2, p. 20-26). For example in the field of anti-parasite vaccines, protection against e.g. *Plasmodium yoelii* has been obtained with DNA-vaccination with the *P. yoelii* circumsporozoite gene (Hoffman, S. *et al.* 1994, Vaccine, vol. 12, p. 1529-1533), and protection against *Leishmania major* has been obtained with DNA-vaccination with the *L. major* surface glycoprotein gp63 gene (Xu & Liew 1994, Vaccine, vol. 12, p. 1534-1536).

Such a DNA vaccination can be performed with a nucleic acid, a cDNA fragment, or preferably with a recombinant DNA molecule according to the invention.

Therefore, one preferred embodiment relates to a vaccine according to the invention, characterised in that it comprises a nucleic acid, a cDNA fragment, or a recombinant DNA molecule according to the invention.

Alternatively, a vaccine according to the invention can comprise live recombinant carriers as described above, capable of expressing the Piroplasmid protein according to the invention or immunogenic fragments of said protein. Such vaccines, e.g. based upon a bacterial, a parasitic or a viral carrier or vector have the advantage over subunit vaccines that they better mimic the natural way of infection by Piroplasmida. Also the presentation of the antigens by cells infected with the carriers resembles the route a Piroplasmid protein according to the invention or immunogenic fragments of said protein are presented to the immune system in a natural infection. Moreover, their self-propagation is an advantage since only low amounts of the recombinant carrier are necessary for immunisation.

Thus, another preferred embodiment relates to a vaccine according to the invention, which comprises a live recombinant carrier and a pharmaceutically acceptable carrier.

The host cells as described above can be used to express a Piroplasmid protein according to the invention or an immunogenic fragment of said protein as an expression system. After expression the proteinacious product may be harvested, but alternatively the culture medium or the complete host cells themselves may be used in a vaccine. This has the benefit of omitting purification steps, but of course requires some tolerance by the target mammalians for the media components and/or components of the host cells.

Also within the scope of the invention is a vaccine according to the invention comprising a combination of two or more types of molecules from the Piroplasmid protein according to the invention or an immunogenic fragment of said protein, or a nucleic acid, cDNA, recombinant molecule, live recombinant carrier, or host cells according to the invention. For such vaccines according to the invention the components may be combined in a single dose or in separate doses, and these may be given at the same time or sequentially.

For instance, a combination vaccination of an initial priming with a recombinant DNA plasmid carrying the coding sequence of a Piroplasmid protein, followed some time later by a booster vaccination with a Piroplasmid protein may advantageously be used.

Vaccines according to the invention, can be administered in amounts containing between 0.1 and 1000 µg of a Piroplasmid protein according to the invention or an immunogenic fragment of said protein per mammalian target. Smaller or larger doses can

in principle be used; preferably a dose of between 50 and 200 µg of a Piroplasmid protein or an immunogenic fragment thereof is used.

For live viral vector vaccines the dose rate per animal may range from 1 to 10^{10} pfu, preferably 10 – 10^5 pfu are used.

A pharmaceutically acceptable carrier is understood to be a compound that does not adversely effect the health of the animal to be vaccinated, at least not to the extend that the adverse effect is worse than the effects seen when the animal would not be vaccinated. A pharmaceutically acceptable carrier can be e.g. sterile water or a sterile physiological salt solution. In a more complex form the carrier can e.g. be a buffer.

Often, a vaccine is mixed with stabilizers, e.g. to protect degradation-prone components from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilizers are i.a. SPGA (Bovarnik *et al.* 1950, J. Bacteriology, vol. 59, p. 509), skimmed milk, gelatine, bovine serum albumin, carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

The vaccine according to the invention may additionally comprise a so-called "vehicle". A vehicle is a compound to which the proteins, protein fragments, nucleic acids or parts thereof, cDNA's, recombinant molecules, live recombinant carriers, and/or host cells according to the invention adhere, without being covalently bound to it. Such vehicles are i.a. bio-microcapsules, micro-alginates, liposomes, macrosols, aluminium-hydroxide, -phosphate, -sulphate or -oxide, silica, Kaolin®, and Bentonite®, all known in the art.

An example is a vehicle in which the antigen is partially embedded in an immune-stimulating complex, the so-called ISCOM® (EP 109.942, EP 180.564, EP 242.380).

In addition, the vaccine according to the invention may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Span® or Tween®.

Target subjects for the vaccine according to the invention are preferably mammalian, e.g. humans or mammalian animals of veterinary importance. The target may be healthy or diseased, and may be seropositive or -negative for Piroplasmidal parasites or for antibodies to Piroplasmidal parasites. The target subject can be of any age at which it is susceptible to the vaccination.

The more preferred target mammalians for the vaccine according to the invention are bovines, equines, canines, and felines.

The vaccine according to the invention can equally be used as prophylactic and as therapeutic treatment, and interferes with the establishment and/or with the progression of an infection or its clinical symptoms of disease.

Therefore one aspect of the invention relates to the use of a nucleic acid sequence according to the invention, a cDNA fragment according to the invention, a recombinant DNA molecule according to the invention, a live recombinant carrier according to the invention, or a host cell according to the invention for the manufacture of a vaccine for prophylactic or therapeutic treatment of an infection or its clinical signs caused by a Piroplasmid organism.

The vaccine according to the invention prevents or reduces the spread of Piroplasmid infection through the population or to the environment.

The vaccine according to the invention can be in several forms, e.g.: a liquid, a gel, an ointment, a powder, a tablet, or a capsule, depending on the desired method of application to the target.

Preferably the vaccine is in the form of an injectable liquid.

The vaccine according to the invention can be administered to the mammalian target according to methods known in the art. For instance by parenteral applications such as through all routes of injection into or through the skin: e.g. intramuscular, intravenous, intraperitoneal, intradermal, submucosal, or subcutaneous. Alternative routes of application that are feasible are by topical application as a drop, spray, gel or ointment to the mucosal epithelium of the eye, nose, mouth, anus, or vagina, or onto the epidermis of the outer skin at any part of the body; by spray as aerosol, or powder. Alternatively, application can be via the alimentary route, by combining with the food, feed or drinking water e.g. as a powder, a liquid, or tablet, or by administration directly into the mouth as a liquid, a gel, a tablet, or a capsule, or to the anus as a suppository.

The preferred application route is by intramuscular or by subcutaneous injection.

It goes without saying that the optimal route of application will depend on the specific particularities of the parasitic infection or clinical disease that is to be prevented or ameliorated, on the characteristics of the vaccine formulation that is used, and on particular characteristics of the target species.

The scheme of the application of the vaccine according to the invention to the target mammalian can be in single or multiple doses, which may be given at the same time or sequentially, in a manner compatible with the dosage and formulation, and in such an amount as will be immunologically effective.

The vaccines of the invention are advantageously applied in a single yearly dose.

In a preferred embodiment, the vaccine according to the invention is characterised in that it comprises an adjuvant.

An adjuvant in general is a substance that boosts the immune response of the target in a non-specific manner. Many different adjuvants are known in the art. Examples of adjuvants are Freund's Complete and -Incomplete adjuvant, vitamin E, non-ionic block polymers and polyamines such as dextransulphate, carbopol and pyran. Also very suitable are saponins, which are the preferred adjuvants. Saponins are preferably added to the vaccine at a level between 10 and 10.000 μg/ml. Within the group of saponins, the saponin Quil A® is the more preferred adjuvant. Saponin and vaccine components may be combined in an ISCOMS® (EP 109.942, EP 180.564, EP 242.380).

Furthermore, peptides such as muramyldipeptides, dimethylglycine, tuftsin, are often used as adjuvant, and mineral oil e.g. Bayol® or Markol®, vegetable oils or emulsions thereof and DiluvacForte® can advantageously be used.

It goes without saying that other ways of adjuvating, adding vehicle compounds or diluents, emulsifying or stabilizing a vaccine are also within the scope of the invention. Such additions are for instance described in well-known handbooks such as: "Remington: the science and practice of pharmacy" (2000, Lippincot, USA, ISBN: 683306472), and: "Veterinary vaccinology" (P. Pastoret *et al.* ed., 1997, Elsevier, Amsterdam, ISBN 0444819681).

The vaccine according to the invention can advantageously be combined with another antigen, or with an immunoactive component. This can also be added in the form of its encoding nucleic acid.

Therefore, in a more preferred embodiment the vaccine according to the invention is characterised in that it comprises an additional immunoactive component or a nucleic acid encoding said additional immunoactive component

The additional immunoactive component(s) may be an antigen, an immune enhancing substance, and/or a vaccine; either of these may comprise an adjuvant.

The additional immunoactive component(s) when in the form of an antigen may consist of any antigenic component of human or veterinary importance. It may for instance comprise a biological or synthetic molecule such as a protein, a carbohydrate, a lipopolysacharide, a nucleic acid encoding a proteinacious antigen, or a recombinant nucleic acid molecule containing such a nucleic acid operably linked to a transcriptional regulatory sequence. Also a host cell comprising such a nucleic acid, a recombinant nucleic acid molecule, or an LRC containing such a nucleic acid, may be a way to deliver the nucleic acid or the additional immunoactive component. Alternatively it may comprise a fractionated or killed microorganism such as a parasite, bacterium or virus.

The additional immunoactive component(s) may be in the form of an immune enhancing substance e.g. a chemokine, or an immunostimulatory nucleic acid, e.g. a CpG motif. Alternatively, the vaccine according to the invention, may itself be added to a vaccine.

For instance a vaccine according to the invention can be combined with a preparation of a Babesia subunit vaccine protein, not being a Piroplasmid protein according to the invention or an immunogenic fragment of said protein, to form a combination subunit vaccine against Piroplasmidal infection or associated clinical signs of disease.

Alternatively, the vaccine according to the invention can advantageously be combined with a pharmaceutical component such as an antibiotic, a hormone, or an anti-inflammatory drug.

In an even more preferred embodiment, the vaccine according to the invention is characterised in that said additional immunoactive component or nucleic acid encoding said additional immunoactive component is obtained from an organism infective to: canines: *Ehrlichia canis, Babesia gibsoni, B. vogeli, B. rossi, Leishmania donovani*-complex, Canine parvovirus, Canine distemper virus, *Leptospira interrogans serovars canicola, icterohaemorrhagiae, pomona, grippotyphosa, bratislava,* Canine hepatitis virus, Canine parainfluenza virus, rabies virus, *Hepatozoon canis* and *Borrelia burgdorferi;* to bovines: Bovine Herpes virus, Bovine Viral Diarrhoea virus, Parainfluenza type 3 virus, Bovine Paramyxovirus, Foot and Mouth Disease virus, *Pasteurella haemolytica*, Bovine Respiratory Syncytial Virus, *Theileria* sp., *Babesia* sp., *Trypanosoma* sp., *Anaplasma* sp., *Neospora caninum, Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma, E.*

coli, Enterobacter, Klebsiella, Citrobacter, Cryptosporidium, Salmonella and *Streptococcus dysgalactiae*; and to

equines: *Streptococcus equi, Streptococcus zooepidemicus, Rhodococcus equi, Corynebacterium pseudotuberculosis, Pseudomonas mallei, Actinobacillus equili* and *Pasteurella multocida*. Potomac fever agent, *Clostridium tetanii, Mycobacterium pseudomallei*, Vesicular Stomatitisvirus, Borna disease virus, Equine influenza virus, African horse sickness virus, Equine arteritis virus, Equine herpes virus 1-4, Infectious anaemia virus, Equine encephalomyelitis virus and Japanese B encephalitis virus.

The Piroplasmid protein according to the invention, or the immunogenic fragment of said protein, the nucleic acid, cDNA, recombinant molecule, live recombinant carrier, and/or the host cells according to the invention for the first time allow the efficient generation of specific antibodies against a Piroplasmid protein, or an immunogenic fragment of said protein. This makes the vaccine according to the invention suitable as marker vaccine, as it allows the differentiation between parasite infected and -vaccinated mammalian targets, through methods known in the art.

Alternatively, these specific antibodies may be used as a vaccine themselves, for so called "passive vaccination".

Therefore another aspect of the invention relates to a vaccine, characterised in that it comprises an antibody against a protein according to the invention, or an antibody against an immunogenic fragment of said protein, or a combination thereof, and a pharmaceutically acceptable carrier.

The antibody may be of natural or synthetic origin. The antibody may be in the form of an antiserum or a purified antibody. Such purified antibodies can advantageously be obtained from an expression system.

Methods for large-scale production of antibodies according to the invention are also known in the art. Such methods rely on the cloning of (fragments of) the genetic information encoding the protein according to the invention in a filamentous phage for phage display. Such techniques are described i.a. at the "Antibody Engineering Page" under "filamentous phage display" at

http://aximt1.imt.uni-marburg.de/~rek/aepphage.html., and in review papers by Cortese, R. *et al.*, (1994) in Trends in Biotechn., vol. 12, p. 262-267; by Clarckson, T. & Wells, J.A. (1994) in Trends in Biotechn., vol. 12, p. 173-183; Marks, J.D. *et al.*, (1992) J. Biol. Chem., vol. 267, p. 16007-16010; Winter, G. *et al.*, (1994) Annu. Rev. Immunol., vol. 12, p. 433-455, and by Little, M. *et al.*, (1994) Biotechn. Adv., vol. 12, p. 539-555.

The phages are subsequently used to screen camelid expression libraries expressing camelid heavy chain antibodies. (Muyldermans, S. and Lauwereys, M., Journ. Molec. Recogn., vol. 12, 131-140 (1999) and Ghahroudi, M.A. *et al.*, FEBS Letters, vol. 414, p. 512-526 (1997)). Cells from the library that express the desired antibodies can be replicated and can subsequently be used for large-scale expression of antibodies.

A combination in a vaccine of an antigen 'loaded' with antibodies against that antigen is known in the art as a "complex" vaccine. Such vaccines according to the invention may advantageously be used.

For reasons of e.g. stability or economy the Piroplasmid protein according to the invention or immunogenic fragments of said protein, or nucleic acids, cDNA's, recombinant molecules, live recombinant carriers, host cells or vaccines according to the invention may be freeze-dried. In general this will enable prolonged storage at temperatures above zero ° C, e.g. at 4°C.

Procedures for freeze-drying are known to persons skilled in the art; equipment for freeze-drying at different scales is available commercially.

Therefore, in a most preferred embodiment, the vaccines according to the invention are characterised in that said vaccines are in a freeze-dried form.

To reconstitute a freeze-dried vaccine, it may be suspended in a physiologically acceptable diluent. Such a diluent can e.g. be as simple as sterile water, or a physiological salt solution. In a more complex form it may be suspended in an emulsion as outlined in PCT/EP99/10178.

Still another aspect of the invention relates to a method for the preparation of a vaccine according to the invention, said method comprising the admixing of a protein according to the invention or an immunogenic fragment of said protein, a nucleic acid, a cDNA fragment, a recombinant DNA molecule, a live recombinant carrier, or a host cell according to the invention, or a combination thereof, and a pharmaceutically acceptable carrier.

Yet another aspect of the invention relates to a method for the preparation of a vaccine according to the invention, said method comprising the admixing of an antibody against a protein according to the invention or an antibody against an immunogenic

fragment of said protein, or a combination thereof, and a pharmaceutically acceptable carrier

As outlined above, a vaccine obtainable by the methods according to the invention can equally be used as prophylactic and as therapeutic treatment, and will interfere both with the establishment and/or with the progression of an infection or its clinical signs of disease.

Therefore, a further aspect of the invention relates to the use of a protein according to the invention or an immunogenic fragment of said protein, for the manufacture of a vaccine for prophylactic or therapeutic treatment of an infection or its clinical signs caused by an organism of the Piroplasmida.

Again a further aspect of the invention relates to a diagnostic test for the detection of a nucleic acid associated with a Piroplasmid organism, characterised in that the test comprises a nucleic acid, said nucleic acid being at least 70 %, preferably 75 %, more preferably 80, 85, 90, 92, 94, 95, 96, 97, 98, 99, or 100 % in that order of preference, similar to the nucleic acid sequence depicted in SEQ ID NO: 1, 3, 5, 7, or 9 or a nucleic acid that is complementary to said nucleic acid, wherein either of the nucleic acids have a length of at least 15 nucleotides, preferably 17, more preferably 18, 19, 20, 24, 28, 32, 35 or 40 nucleotides, in that order of preference.

Yet a further aspect of the invention relates to a diagnostic test for the detection of antibodies against a Piroplasmid organism, characterised in that said test comprises a protein according to the invention or an immunogenic fragment of said protein, or a combination thereof.

For instance BIIA1 or BIIA2 or an immunogenic fragment of either is coupled to a solid phase carrier, this is incubated with a sample to be tested, is washed, and presence of bound antibodies is detected. Preferred diagnostic method is by ELISA.

Still a further aspect of the invention relates to a diagnostic test for the detection of antigenic material from a Piroplasmid organism, characterised in that said test comprises an antibody against a protein according to the invention or an antibody against an immunogenic fragment of said protein, or a combination thereof.

For instance antibodies against BIIA1 or BIIA2 or an immunogenic fragment of either are coupled to a solid phase carrier, this is incubated with a sample to be tested, is washed, and presence of bound protein is detected. Preferred diagnostic method is by ELISA.

5

The invention will now be further described with reference to the following, non-limiting, examples.

EXAMPLES

EXAMPLE I

1.1. TECHNIQUES USED

1.1.1. *B. bovis in vitro* culture

B. bovis Israel isolate (clonal line C61411) was cultured *in vitro* as previously described (Levy & Ristic 1980, Science, vol. 207, p. 1218-1220). Briefly, *B. bovis* cultures were maintained in 24-well plates (1.2 ml total volume) or in 25 cm^2 bottles (15 ml total volume) containing medium M199 (Cambrex Bioscience, Belgium), with 40% bovine serum (from an adult donor cow), 50 $\mu g ml^{-1}$ Gentamicin (Gibco BRL), 25 mM sodium bicarbonate, and bovine erythrocytes at 5% packed cell volume (PCV). Cultures were incubated at 37°C, 5% CO_2 in air, and parasitaemia was kept between 1% and 12% by daily dilution.

B. bovis Mexico isolate (clonal line C9.1) was cultured according to the same protocol as used for clonal line C61411 (Israel isolate) except that cultures were maintained at 90% N_2, 5% CO_2, 5% O_2 instead of 5% CO_2 in air.

1.1.2. Construction of *B. bovis* genomic and cDNA library

A cDNA library was constructed from 5 µg *B. bovis* mRNA using the λZAP-cDNA® Synthesis Kit (Stratagene) according to the manufacturer's instructions. cDNA fragments of 0.5 to 4 kb were collected by gel filtration on a sepharose CL4B column and ligated into the *Eco*RI / *Xho*I site of λ uniZAP-XR Express vector. Giga pack III Gold was used for packaging into phage particles followed by transformation of *Escherichia coli* XL-1 Blue MRF'cells. 1.2×10^6 plaques were obtained of which an amplified library was made.

Single-pass sequence runs were performed on 15000 cDNA clones that were automatically picked at random from the plated cDNA library to establish an EST dataset. From this EST dataset a database consisting of 12892 high quality sequences (476 bp average length) was constructed.

For constructing the genomic library, 600 µg of *B. bovis* DNA was partially digested with EcoRI (150 units or 250 units) for 1 h at 37°C. The digested DNA was size fractionated on a Sepharose CL-4B column. Fragments of 0.5 kb to 8 kb were ligated into the EcoRI site of λ-ZAPII-Express, packaged using Gigapack III Gold Packaging extract

and transformed in *E. coli* XL1-Blue MRF'competent cells. 2.5×10^6 plaques were obtained of which an amplified library was made.

The cDNA libraries were screened with a probe produced through PCR with primers specific for BIIA1 or for BIIA2.

1.1.3. screening of *B. bovis* genomic and cDNA library for the genes for BIIA1 and BIIA2

The *B. bovis* genomic and cDNA libraries were screened to isolate clones for the genes of BIIA1 and BIIA2 with a specific probe made by PCR. Specific primers used were: for the BIIA1 gene:

primer 1: 5'- CCACGGCTCTGGAATCTATGTC -3' (SEQ ID NO: 11)
primer 2: 5'- CAAAAGGATACCTATATTTGGTAC -3' (SEQ ID NO: 12),

and for the BIIA2 gene:

primer 3: 5'- TGTGGTAGATGAATCTGCTAGTATATC -3' (SEQ ID NO: 13)
primer 4: 5'- CTATGCCACGGCATTCAGCAACATTTA -3' (SEQ ID NO: 14)

Both primer pairs were used to amplify a fragment from a clone from the EST database of *B. bovis*, by PCR in a 50 µl volume containing 0.2 mM dNTP, 20 pmol/µl of each primer, 100 ng *B. bovis* total genomic DNA and 0.5 U Taq DNA polymerase in standard buffer (Promega). Amplification was performed for 30 cycles with the conditions for the BIIA1 probe at: 92°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and for the BIIA2 probe at: 95°C for 1 min, 58°C for 1 min, and 72°C for 10 min. These cycles were preceded by initial denaturation for 3 min at 95°C and a final elongation at 72°C for 10 min.

Both probes were purified from agarose gel and labelled with 50 µCi ^{32}P-dATP (3000 Ci/mmol), using a Random Primer labelling kit (Roche). In total 4.10^6 cDNA and 4.10^5 genomic DNA library plaques were screened by standard procedures (Sambrook & Russell, supra) for cloning the BIIA1 cDNA; whereas 5.10^5 cDNA and an equal number of genomic DNA library plaques were screened for cloning the BIIA2 cDNA. After 2 cycles of plaque purification all clones were *in vivo* excised for isolation of the phagemid inserts as described in the manufacturer's instructions (Stratagene) and sequenced on both strands, using automated cycle sequencing with the dye terminator method (ABI PRISM® dye terminator kit, Pharmacia).

To obtain the full-length BIIA1 and BIIA2 cDNA's, the non-coding 5'-ends were identified with 5'-RACE (GeneRacer™ kit, Invitrogen; L1502-01, according to the manufacturer instructions). The obtained full length clones were inserted into pCR2.1 cloning plasmids and sequenced on both strands, as described above. The resulting sequences are presented in SEQ ID NO: 1 (BIIA1) and SEQ ID NO: 5 (BIIA2).

1.1.4. Expression of recombinant BIIA1 in *E. coli*

The clones of BIIA1 en BIIA2 were subcloned by PCR from the pCR2.1 cloning plasmids.

The primers used for subcloning BIIA1 were:

primer 5: 5'- CCCGGATCCATGCAGTTACATAACAAA -3' (SEQ ID NO: 15)

primer 6: 5'- GGGAAGCTTCTGAGCAAAGGAAATAGG -3' (SEQ ID NO: 16)

These primers for BIIA1 introduced a *Bam*HI restriction enzyme site prior to base 1 (numbered from the first base of the initiation codon) and a *Hin*dIII site after base 1504.

The primers used for subcloning BIIA2 were:

primer 7: 5'- CCCGAATTCGTGGTAGATGAATCTGCT -3' (SEQ ID NO: 17)

primer 8: 5'- CCCGTCGACTGCCTCGCCCCAAATGTTGT -3' (SEQ ID NO: 18)

These primers for BIIA2 introduced an *Eco RI* site, and a *Sal I* site.

After PCR (30 cycles of 1 min 94°C, 1 min 55°C, 1 min 72°C), the fragments were gel purified, annealed to pET-32a vector and used for transformation in *E. coli* NovaBlue® strain. Plasmids containing the appropriate insert were used to transform in expression host strains, BL21 (DE3). Fusion proteins with thioredoxin were obtained with maximal yield after induction with 1 mM of isopropyl-β-D thiogalactosidase (IPTG) for 4 hr at 37°C as shown by analysis of total cell samples at 0 and 4 hr after induction. Bacterial pellets were boiled at 95°C in SDS-polyacrylamide (SDS-PAGE) sample buffer containing 2% (v/v) β-mercaptoethanol, run on 10% SDS-PAGE minigels, and Coomassie Brilliant Blue stained to confirm expression (Figures 1 and 2).

1.1.5. Peptide selection and generation of monospecific antiserum

After the BIIA1 and BIIA2 genes were completely sequenced, peptides were selected from computer-translated sequences, for induction of specific polyclonal antibodies through immunisation of test animals.

The sequence analysis program Protean of DNA Star® was used to select peptide regions that have a good surface probability and contained charged alpha amphiphathic regions.

Peptides selected from BIIA1 (SEQ ID NO: 2) were:

peptide 1: aa numbers 46-60:	cysteine-AFHKEPNNRRLTKRS,
peptide 2: aa numbers 395-409:	cysteine-RGVGMNWATYDKDSG,
peptide 3: aa numbers 453-467:	cysteine-YVEPRAKNTNKYLDV.

Peptides selected from BIIA2 (SEQ ID NO: 6) were:

peptide 4: aa numbers 255-269:	cysteine-PGKRTRALLDLRMIE ,
peptide 5: aa numbers 424-439:	cysteine-RVGNTDEEHNHRKDMD,
peptide 6: aa numbers 547-561:	cysteine-VYDDHPEESENTGIN.

After the synthesis of the peptides, they were coupled to a carrier protein: Maleimide activated keyhole limpet haemocyanin (KLH) (Pierce; 77605) according to the manufacturer's instructions. The peptide-carrier conjugate was used to generate rabbit polyclonal antisera.

For that purpose three groups of NZW-rabbits (each group contained 2 rabbits) were immunized five times subcutaneously with a 3-week interval between consecutive immunizations. Before the immunisation blood serum was collected of each rabbit, which was used as negative control. Each rabbit was injected with 250 µg peptide coupled to KLH that was taken up in an equal volume of adjuvant Stimune® (ID-DLO, Lelystad, the Netherlands). Total volume that was injected in each rabbit was 1000 µl. Sera were tested periodically for reactivity by ELISA. Plasmaforeses were done one week after the last immunization and sera were collected.

1.1.6. ELISA

The antibody response was evaluated by ELISA. Ninety-six-well microtiter plates were coated with 150 ng of either peptide 1 or peptide 2 per well, incubated 30 min at 37°C, blocked for 1 h with PBS/BSA. Consecutive dilutions (1:50 to 1:50.000) of individual rabbit sera were incubated for 1 h at 37 °C. The plates were washed, and 1:2000 diluted swine anti rabbit HRP-conjugated secondary antibody was incubated for 1h. The plates were

washed and developed for 45 min with ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)]- peroxidase substrate (Roche biochemicals). The OD_{405} was recorded, and comparative ELISA titres were calculated.

1.1.7. Immunofluorescence assay

The recognition of *B. bovis* merozoites by anti-sera against peptides from BIIA1 and BIIA2 was tested by indirect immunofluorescence assay (IFA). Thin blood smears were fixed with chilled methanol. Primary incubation with polyclonal rabbit anti-BIIA1 (1:40) or polyclonal mouse anti-BIIA1 (1:5 to 1:160) for 30 min was followed by three wash steps of 5 min. Slides were incubated with 1:80 goat anti-rabbit immunoglobulin G (IgG) fluorescein isothiocyanate- labelled antibodies (Nordic) for 30 min. The slides were washed again, and Vectashield® solution (Vector laboratories) was applied, objects were covered with a cover-glass and visualized on a UV fluorescence microscope with FITC filters (450-480/ 515-565 nm). IFA titres were determined as the last serum dilution with a positive recognition of the parasite compared to the negative pre-immune serum diluted 1:5.

1.1.8. Preparation of total merozoite protein extracts and proteins solubilised upon invasion

800 µl samples of merozoites, prepared as described above for *in vitro* invasion, were partially separated from erythrocyte ghosts by filtration over 1.2 µM polypropylene prefilters (Millipore, AN1202500). Filtered merozoites were pooled and washed twice in 20 volumes of PBS containing 25 mM sodium bicarbonate (pH 8.0) followed by centrifugation at 2000 g for 20 min at 4°C. After the second wash the pellet was resuspended in an equal volume of PBS (pH 8.0) and divided in aliquots of 200 µl that were centrifuged (10.000xg, 5 min at 4°C) and stored as 100 µl cell pellets (2×10^9 merozoites) at -20°C after removal of supernatant. Frozen merozoite pellets were thawed just before use and lysed, reduced and alkylated by using a Proteoprep® membrane extraction kit (Sigma) according the manufacturer's instructions and finally obtained in 1.7 ml of buffer compatible with direct application on SDS-polyacrylamide gels or iso-electrofocussing (IEF) strips. Insoluble material was removed by centrifugation at 16.000×g for 3 min at 4°C. Protein concentration was determined by the Bradford method (Anal. Biochem. 1976, vol. 72, p. 248-254). As the extracts contained considerable amounts of erythrocyte proteins, control extracts were prepared in the same way but starting with a culture of non-infected erythrocytes.

Proteins solubilised upon invasion were obtained by gently removing the overlaying buffer after 1 h of *in vitro* invasion as described above. The samples were centrifuged (2000xg, 10 min, 4 °C) after which the pellet (which was invisible) was discarded and the supernatant centrifuged again at high speed for removal of membrane fragments (20 min, 12.000xg, 4°C). The final supernatant was dialysed (Pierce; Snakeskin® pleated dialysis tubing, 68035) overnight against 10 mM $KHPO_4$, pH 7.5. Residual haemoglobin was removed batchwise by incubating 50 ml of the dialysed supernatant with 6.5 ml DEAE sepharose fast flow (Amersham Biosciences) equilibrated in dialysis buffer for 90 min at 4°C on a rotating platform. The suspension was centrifuged for 5 min at 3000xg at 4°C after which the DEAE sepharose was washed 4 times by addition of 50 ml of dialysis buffer followed by centrifugation for 5 min at 3000xg at 4°C. Bound proteins were eluted by addition of 6 ml of elution buffer (350 mM KCl, 10 mM $KHPO_4$, pH 7.5) and incubation for 5 min followed by centrifugation for 5 min at 3000xg at 4°C. The supernatant was concentrated and de-salted over 10 kDa filters (YM-10, Millipore).

1.1.9. SDS-polyacrylamide electrophoresis and Western blotting

Proteins were resolved in the presence or absence of β-mercaptoethanol and were separated on a 10% SDS-PAGE and electrophoretically transferred to an Immobilon™-P membrane (Millipore). The blot was blocked with 5% skimmed milk diluted in 0.5% Tween® 20 containing phosphate-buffered saline (PBST) for 1h at 37°C. An appropriate dilution (1:500) of primary antibody in 2% skimmed milk in PBST was incubated for 1 h overnight. The blot was washed with PBST and then incubated with a 1:10.000 dilution of anti- rabbit- horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO) for 1 h at 37°C. After being washed with PBST, the blot was developed with TMB MB substrate kit (Lucron Bioproducts BV; KPL 50-77-00) or with enhanced chemoluminescence (ECL)+ (Amersham; RPN2132).

1.1.10. Iso-electric focusing

Total merozoite extract, invasion supernatant, and BIIA1 protein samples were resuspended in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 2% carrier ampholyte mixture pH 4-7NL (IPG buffer and 20 mM DTT). BIIA2 protein samples were separated in the first dimension using carrier ampholyte mixture pH 3-10NL. IEF instrumentation, IPG gels and reagents used were from Amersham Biosciences, unless

otherwise indicated. 35 µg total merozoite protein or 35 µg invasion supernatant with protease inhibitor (Complete, Roche) was loaded on 7 cm strips (pH 4-7NL). For 13 cm strips, 150 µg of total merozoite proteins or 150 µg invasion supernatant was loaded. Strips were rehydrated (10-14 h) and focused overnight (14-17 h) in an automated run (1 min 300 V, 90 min during which the voltage rose to 3500 V, followed by continued focusing at 3500 V, to a total of 35-40 KVh, on IPGPhor™).

Following iso-electric focussing, the proteins were reduced and bound to SDS by equilibrating each strip for 15 min in 10 ml of SDS equilibration buffer (50mM Tris, 6M urea, 2% SDS, 30% glycerol, pH 8.8) containing 30 mM DTT (added fresh before use). A second equilibration step in SDS equilibration buffer containing 2.5% iodoacetamide (also freshly added) instead of dithiotreitol, was performed in order to prevent protein reoxidation and to minimise reactions of cysteine residues.

The second-dimensional SDS gel electrophoresis gel was carried out in a Hoefer SE600 system. Silver staining was used to visualise proteins after 2-D electrophoresis. Images of the gels were acquired using LabScan® v3.0 software on a Umax flatbed scanner and were analysed using ImageMaster® 2D v3.01 software (Amersham Biotech). For immune blotting, proteins on 7 cm strips were separated on a 10% SDS-PAGE gel or 13 cm strips were separated on 2-D protein gel and transferred to an Immobilon™-P membrane (Millipore; IPVH00010). The procedure followed for two-dimensional blots was the same as that for the 1-D blots.

1.1.11. *B. bovis in vitro* invasion assay

Invasion was performed as described previously (Fransen *et al.* 2003, Microbes Infect. vol. 5, p. 365-372), with slight modifications. *B. bovis* infected red blood cells at 6 to 8% parasitaemia, were centrifuged at 2000xg, 10 min, 15°C, and resuspended in an equal volume of VyMs buffer (Vega & Martinez, see Fransen, supra). 800 µl samples were submitted to five intermittent (10 seconds, at 0°C in between pulses) high voltage pulses (2.5 kV, 200Ω, 25 µF) in 4 mm BioRad cuvettes (165-2088) using a BioRad Gene Pulser® with pulse controller.

8 ml of PBS containing 25 mM sodiumbicarbonate (pH 8.0, 20°C) was added to each 800 µl sample followed by centrifugation (1800xg) for 10 min at 15°C. A second, identical wash was performed except that centrifugation was done at 1300xg after which the merozoite pellet was resuspended in 800 µl PBS containing 25 mM sodiumbicarbonate (pH 8.0, 20°C). Invasion was initiated by addition of 1 volume of

resuspended merozoites to 9 volumes of suspended bovine erythrocytes (5.5% PCV in PBS pH 8.0 containing 25 mM sodiumbicarbonate, pre-incubated for 30 min at 37°C in CO_2 in air) and was performed in 24-well plates (final volume 1.2 ml), in 25-cm^2 flasks (15 ml) or in 80 cm^2 flasks (50 ml) at 37°C, 5% CO_2 in air. Giemsa-stained slides were prepared after 1 h and parasitisised erythrocytes out of a total of 5000 erythrocytes were counted.

1.1.12. *In vitro* inhibition of invasion by polyclonal rabbit antisera

200 µl of *B. bovis* merozoites, liberated by high voltage pulsing and resuspended in PBS containing 25 mM sodiumbicarbonate (pH 8.0) as described above, were incubated with 40 µl of rabbit antisera for 1 h at 20°C. After 1 h, 960 µl of suspended bovine erythrocytes (6.25% PCV in PBS pH 8.0 containing 25 mM sodiumbicarbonate, pre-incubated for 30 min at 37°C in CO_2 in air) were added, followed by 1 h of incubation after which Giemsa-stained slides were prepared and counted to determine the level of invasion. The rabbit antisera used were raised against synthetic peptides derived from the BIIA1 and BIA2 amino acid sequence and a control serum raised against an unrelated control peptide (YAGRLFSKRTAATAYKLQ). Peptides had been linked to keyhole limpet haemocyanin (KLH) prior to immunization. Pre-immune sera were also included in the test.

1.2. Results of Example 1

1.2.1. Identification and cloning of a full length cDNA encoding BIIA1 and BIIA2

Probing the *B. bovis* cDNA library with PCR probes (350 bp for BIIA1 and 450 bp for BIIA2), resulted in the cloning and sequencing of a 2181 bp cDNA for BIIA1 and of 2385 bp for BIIA2. Both contained an open reading frame and a 3' non-coding region terminating in a polyA-tail. To determine the 5' capped end of the full-length mRNA's, total mRNA was dephosphorylated after which the 5' caps, which are left intact, were removed by tobacco acid pyrophosphatase followed by ligation of a specific RNA oligonucleotide. Subsequently, nested PCR on first strand cDNA allowed the cloning and sequencing of a fragment representing the 5' end of the *B. bovis* mRNA for BIIA1 and for BIIA2.

Translation by computer of the 1815 bp ORF of BIIA1 predicted a 67.2 kDa; translation of the 1965 bp ORF for BIIA2 predicted a 65.6 kDa protein.

1.2.2. Recognition of recombinant BIIA1 and BIIA2 by antisera against derived peptides.

To enable further studies on the BIIA proteins, rabbits were immunized with KLH-linked synthetic peptides 1 – 6 (*supra*). All antisera specifically recognized a recombinant fusion product of thioredoxin and the part of the BIIA proteins that was expressed in *E. coli* BL21 cells (Figures 1 and 2). Polyacrylamide gel electrophoresis of total cell lysates obtained before (lane 1) and after (lane 2) induction with IPTG identified the recombinant fusion product for BIIA1 and for BIIA2. Rec BIIA1 and BIIA2 both are recognized by all three immune sera (lanes 5, 8, 11) and not by pre-immune sera (lanes 6, 9, 12) on immuno-blots. Immune recognition was specific for the BIIA part of the fusion product as a control protein, a recombinant fusion product of *B. bovis* rab5 (lane3, Asp-5 to Lys-208, GenBank Acc. No.: 324137.1) expressed in PET32a was not recognized (lanes 7, 10, 13) by these sera. Also, immune recognition was peptide specific and not due to antibodies induced by the KLH carrier protein used for immunization as antiserum raised against a KLH-linked synthetic peptide unrelated to BIIA1 or BIIA2 did not recognize the BIIA1 recombinant fusion product (lane 13).

1.2.3. Immunofluorescence microscopy

To localize the BIIA proteins in the parasite, immunofluorescence studies using rabbit antisera against the six KLH-linked peptides of BIIA1 and BIIA2 were performed on *B. bovis in vitro* cultures attached to glass slides by methanol fixation (Figures 3 and 4). Incubation with pre-immune sera (panels A, C, E) did not result in any specific staining of parasites above a background signal of faint fluorescence derived from infected as well as non-infected erythrocytes. In contrast, immune sera resulted in specific staining of parasites in any microscope field examined (panels B, D, F). Fluorescent parasites were detectable with antisera against all three peptides at a dilution of 1:5. Although intra-erythrocytic *B. bovis* parasites and free merozoites are small (± 1 by 2 μm) a maximal magnification allowed a clear visualization of the staining pattern.

1.2.4. Inhibition of *in vitro* invasion by peptide-specific antisera

A *B. bovis in vitro* invasion assay, allowing the study of the invasion of erythrocytes by free merozoites in a protein free buffer within a time span of 1 h, was used to assess the effect of antisera directed against the 6 peptides derived from different domains of BIIA1 and BIIA2. Free merozoites were pre-incubated for 1 h at 20°C with the anti-peptide antisera and with the control serum directed against a non-related peptide after which

invasion was started by the addition of erythrocytes. All antisera against the BIIA peptides gave rise to significant inhibition of invasion whereas pre-immune sera and control antiserum had no significant effect on invasion efficiency (Figures 5 and 6). For BIIA1, the strongest effect of 65 ± 10 % inhibition of invasion was observed by the antiserum directed against peptide1; for BIIA2, the strongest effect of 70 ± 10 % inhibition of invasion was observed by the antiserum directed against peptide 4.

1.2.5. Mapping BIIA proteins on 2-D-gels

To determine whether BIIA1 and BIIA2 become exposed in the medium as soluble proteins during invasion of erythrocytes, thus constituting part of the SPA mentioned above, immunoblotting of invasion supernatants was performed. BIIA1 and BIIA2 were localized on two-dimensional immunoblots. 50 µg of concentrated invasion supernatant was separated by iso-electrofocussing followed by electrophoresis on SDS-polyacrylamide gels. Proteins were blotted on PVDF membranes. Excised parts of the membranes (45 to 90 kDa) were incubated with anti-BIIA1 peptide antisera against peptides 1 or 3 (Figure 7, panels A and C respectively) as well as with anti-BIIA2 peptide antisera against peptides 4 and 6 (Figure 8, panels A and C respectively). For both proteins, antibodies against peptides 1 and 4, were bound to the same specific spots (arrows) in addition to a-specific staining of proteins that were also present on control blots. These had been prepared from supernatants of uninfected red blood cells (RBC) prepared under identical conditions but in absence of merozoites (Figure 7 and 8, panels B and D). Spots localized by immunoblotting were subsequently matched to a silver-stained 2-D-protein gel of a similar sample that was obtained from a parallel experiment in which use was made of parasites that were metabolically labelled with ^{35}S-Met prior to invasion. Figure 9 displays the pattern obtained after exposure to film showing exclusively proteins of *B. bovis* as erythrocyte proteins have not incorporated label. By using imaging software, the spots detected by immunoblotting with anti-BIIA1-peptide antisera could be matched to a row of ± 70 kDa spots on the autoradiograph and on the silverstained gel (see arrows on Figure 9). BIIA2 is represented by spots of minor intensity indicating a lower abundance of the native protein.

EXAMPLE II: Cloning, expression and characterisation of BIIA3

Total amplified DNA from the *B. bovis* cDNA library described in § 1.1.2 was screened for for the BIIA3 gene with the following primers:

primer 9: 5'- CCCGAATTCCATGATGGTGAAGTTCCACAC -3' (SEQ ID NO: 19)
primer 10: 5'- CCCGTCGACGTTGGCCCCCTTTCGGTGAT -3' (SEQ ID NO: 20)

PCR was performed as described in § 1.1.3.

The PCR fragment was sequenced directly; the resulting sequence is presented in SEQ ID NO: 9 (BIIA3).

The PCR fragment of the BIIA3 cDNA was cloned into expression vetor pET-32a, as described in § 1.1.4. Primers 9 and 10 provided *Eco RI* and *Sal I* restriction sites.

The computer-translated sequence of the BIIA3 protein is presented in SEQ ID NO: 10. The 1635 nucleotide ORF in the BIIA3 cDNA encodes a 61.0 kDa protein.

Peptides were predicted from this protein for induction of specific antibodies in test animals, as described in § 1.1.5.

Peptides selected from BIIA3 protein are:

peptide 7: aa numbers 122 - 136	cysteine - GELKKLSDNIPTKMP,
peptide 8: aa numbers 385 - 399	cysteine - SGSARVETSLESSVP.

The peptides were coupled to KLH, and used to generate rabbit polyclonal antibodies as described in § 1.1.5. Rabbit sera were evaluated by ELISA, as described in §1.1.6.

The rabbit polyclonal anti-petide antisera were to detect recBIIA3 (*E. coli* expressed thioredoxin fused BIIA3 protein) in 1-D Western blot. The results are depicted in Figure 10, panel A: Rec BIIA3 was recognized by antisera against both peptides 7 and 8, whereas preimmune sera did not recognize Rec BIIA3.

Polyclonal antiserum against BIIA3 (and against BIIA1 and BIIA2) was raised in cattle, as described in Example III.

This bovine antiserum was also used in a 1-D Western blot on recBIIA3. Results are depicted in Figure 10, panel B: serum from two animals recognised recBIIA3, whereas pre-immune bovine serum did not.

The bovine antiserum against recBIIA3 was also used on a 2-D gel of native *B. bovis* proteins as described in § 1.1.8 and 1.1.9. Results are shown in Figure 11.

Preimmune bovine serum reacted with several spots of red blood cell origin (panel A). For panel B sepharose column purified recBIIA3-immune IgG was used. This specifically recognised (groups of) spots of ~95 kDa, ~75 kDa and ~ 30 kDa (see arrows). Apparently, processed and multimeric forms of native BIIA3 are also recognised.

The rabbit polyclonal antiserum against peptide 7 was demonstrated to have invasion inhibiting properties, see Figure 12. Sepharose G purified IgG was used at three different concentrations, leading to a maximum inhibition of 65%. Non-immune IgG, and PBS did not result in inhibition (Control column).

Rabbit polyclonal antiserum directed against peptide 7 was also used to determine the subcellular localization of BIIA3 in *B. bovis* merozoites in the infected erythrocyte, by indirect immunofluorescence. Detection was by multiphoton microscopy.

Thin blood smears were fixed in acetone for 10 min and air-dried. Primary incubation with anti peptide 7 rabbit serum (1:20) for 30 min was followed by three wash steps of 5 min with PBS. Slides were then incubated with goat anti-rabbit IgG conjugated with Alexa 488 (20 µg/ml, Molecular Probes Inc., Eugene, USA) for 30 min and washed with PBS. Subsequently, for dual labeling, the slides were incubated with DAPI (0.5 µM, Molecular Probes Inc.) for 20 min and washed. FluorSave® solution was applied and the slides were left overnight at room temperature, covered, in a horizontal position.

Fluorescent signals were visualized using a Bio-Rad Radiance 2100MP confocal and multi-photon system equipped with a Nikon TE300 inverted microscope. Excitation of the DAPI probes was achieved by multi-photon excitation at 780 nm using a mode-locked Titanium-Sapphire laser (Tsunami, Spectra-Physics) pumped by a 10 W solid state laser (Milennia Xs, Spectra-Physics), while the Alexa 488 probe was excited by an Argon laser at 488 nm.

The multiphoton IFT results showed BIIA3 specific staining was present in the apical region of the Babesia parasite.

EXAMPLE III: Generation and use of bovine antisera against recombinant BIIA1, BIIA2, and BIIA3

Recombinant expression products of BIIA1, BIIA2, and BIIA3 were generated in *E. coli* as described in section 1.1.4. Bacteria were pelleted and solubilized in 6M Guanidinium HCl. The total cell lysate was centrifuged at 9000 rpm for 10 min, and the soluble lysate was bound to a suspension of Ni-NTA agarose in GuHCL. Beads were washed three times with 8M Urea, and specific antigen was subsequently eluted with 250 mM imidazol in 3M Urea.

Each vaccine dose contained 100 µg purified recBIIA antigen and was formulated with saponin adjuvant in a 2ml final dose. Vaccines were applied intramuscular in the neck of immunological competent cattle, each group numbered 5 animals. 5 weeks after the priming a booster vaccination was given with the same formulation. 3 weeks after the booster blood was taken and serum was prepared for analysis.

Purification of bovine specific IgG was performed by incubating 5 ml of antiserum with 2 ml of GammaBind Plus® Sepharose (Amersham-biosciences) for 1 h at 20 °C in binding buffer (0.01 M Sodiumphosphate pH 7.4, 0.15 M Nacl, 0.01 M EDTA). The column was washed with binding buffer and IgG was eluated 5 ml 0.5 M NaAc pH 3.0, and immediately neutralised with TrisHCl pH 9.0. IgG was concentrated and dialysed against PBS pH 7.4.

In vitro invasion inhibition by total IgG purified from bovine antisera raised against recombinant BIIA1, BIIA2 and BIIA3 (cloned from Israel strain) was performed as described for polyclonal rabbit antisera (§ 1.1.11 and 1.2.4) using final bovine IgG concentrations of 0.15 µg/µl or 0.75 µg/µl during preincubation. All tests were performed twice using antibodies of two different animals for each antigen. The results shown in Figure 13 display the combined data of the individual antisera per antigen. Standard deviation is indicated. To show the inhibition is also effective on invasion of a heterologous Babesia strain, a clonal line (C9.1) derived from a Mexican isolate (MO7) of *B. bovis* was tested.

The effectivity of the inhibition of erythrocyte invasion by both Babesia strains is comparable. Effectivity of BIIA1 and BIIA2 (between 3 and 12 %) seemed even higher than that of BIIA3 (23 - 25 %).

LEGEND TO THE FIGURES

Figure 1:

Lane 1: pET-BIIA1 before induction with IPTG.

Lane 2: pET-BIIA1 4 h after induction with IPTG.

Lane 3: pET-Rab5 4 h after induction.

Lanes 4, 5, 6 incubated with anti-peptide 1;

Lanes 7, 8, 9 incubated with anti-peptide 2;

Lanes 10, 11, 12 incubated with anti-peptide 3.

Lanes 4, 7, 10 contain pET-BIIA1 4 h after induction, incubated with pre-immune sera;

Lanes 5, 8, 11 the same as in lanes 4, 7, and 10, but incubated with immune sera.

Lanes 6, 9, 12 contain pET-Rab5 4 h after induction incubated with immune sera.

Lane 13: pET-BIIA1 4h after induction, and incubated with antiserum again KLH-linked peptide unrelated to *B. bovis*.

Figure 2:

Lane 1: pET-BIIA2 before induction with IPTG.

Lane 2: pET-BIIA2 4 h after induction with IPTG.

Lane 3: pET-Rab5 4 h after induction.

Lanes 4, 5, 6 incubated with anti-peptide 4;

Lanes 7, 8, 9 incubated with anti-peptide 5;

Lanes 10, 11, 12 incubated with anti-peptide 6.

Lanes 4, 7, 10 contain pET-BIIA2 4 h after induction, incubated with pre-immune sera of rabbits;

Lanes 5, 8, 11 the same as in lanes 4, 7, and 10, but incubated with immune sera.

Lanes 6, 9, 12 contain pET-Rab5 4 h after induction, incubated with immune sera.

Lane 13 contains pET-BIIA2 4h after induction, and incubated with antiserum again KLH-linked peptide unrelated to *B. bovis*.

Figure 3:

Panels A, C and E display methanol-fixed *in vitro* cultures of *B. bovis* incubated with pre-immune rabbit antisera against peptides 1, 2 and 3 of BIIA1 respectively. Panels B, D, F are similar to A, C and E but incubated with the corresponding immune sera. For reproductive purposes the colours have been inverted.

Figure 4:

Panels A, C and E display methanol-fixed *in vitro* cultures of *B. bovis* incubated with pre-immune rabbit antisera against peptide 4, 5 and 6 of BIIA2 respectively. Panels B, D, F are similar to A, C and E but incubated with the corresponding immune sera. For reproductive purposes the colours have been inverted.

Figure 5:

Control columns represent a pre-incubation with antiserum against a non-related peptide that gave no inhibition. Antisera (open bars) as well as pre-immune rabbit sera (black bars) against peptides 1, 2 and 3 of BIIA1 were tested twice in triplo.

Figure 6:

Control columns represent a pre-incubation with antiserum against a non-related peptide that gave no inhibition. Antisera (open bars) as well as pre-immune sera (black bars) against peptides 4, 5 and 6 of BIIA2 were tested twice in triplo.

Figure 7:

Panels A and C: 2-D-immunoblots with immune serum against BIIA1 peptides 1 and 3 respectively. Panels B and D: 2-D-immunoblots with pre-immune serum of rabbits immunized with peptides 1 and 3 of BIIA1 respectively. Arrows indicate spots specific for antisera against peptide 1 as well as peptide 3.

Figure 8:

Panels A and C: 2-D-immunoblots with immune serum against BIIA2 peptides 4 and 6 respectively. Panels B and D: 2-D-immunoblots with pre-immune serum of rabbits immunized with peptide 4 and 6 of BIIA2 respectively. Arrows indicate spots specific for antisera against peptide 4 as well as peptide 6.

Figure 9:

Autoradiograph of a 2-D gel as used for the immunoblots presented in figures 7 and 8, displaying only *B. bovis* derived proteins that were labelled with ^{35}S-Met by metabolic labelling prior to invasion. Arrows indicate the spots that have been identified as BIIA1 by matching with immunoblots shown in figure 7 using imaging software.

Figure 10:

1-D Western blot of *E. coli* expressed recBIIA3, recognized by polyclonal rabbit antisera raised against peptides 7 and 8.

Panel A: rabbit anti-peptide antisera: lane 1: anti-peptide 7; lane 3: anti-peptide 8; both in serum dilution 1:2000.

Lanes 2 and 4: pre-immune sera of both peptide-antisera rabbit donors.

Panel B: Bovine anti-recBIIA3 antisera: lanes 1, and 2: purified immune IgG in 1: 200.000 from two animals; lane 3, pre-immune bovine serum.

Figure 11:

2-D Western blot of native *B. bovis* proteins recognized by bovine polyclonal antiserum directed against recBIIA3.

Panel A: pre-immune bovine serum.

Panel B: Sepharose G purified immune IgG, at 0.8 µg/ml. Arrows indicate BIIA3 specific antibody recognition.

Figure 12:

Invasion inhibition assay of rabbit polyclonal anti-peptide 7 immune IgG, inhibiting the invasion of *B. bovis* Israel isolate into bovine erythrocytes.

Inhibition by control (pre-immune serum) was set to 100 %.

Horizontal axis: concentration of purified immune IgG; vertical axis: relative % of invasion inhibition efficacy, with standard deviation (n=3).

Figure 13:

Invasion inhibition assay of bovine polyclonal immune IgG against *E. coli* expressed recBIIA1, recBIIA2, and recBIIA3, inhibiting the invasion of *B. bovis* isolates from Israel and from Mexico into bovine erythrocytes.

Inhibition by control (pre-immune serum) was set to 100 %.

Horizontal axis: final IgG concentration in µg/µl; vertical axis: relative % of invasion inhibition efficacy, with standard deviation (n=2 x 2).